IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	Slepnev	Examiner:	Horlick, K.
Serial No.:	10/719,185		
Filed:	November 21, 2003	Group Art Unit:	1637
Entitled:	Sampling Method and Apparatus for Amplification Reaction Analysis		
		Conf. No.:	3232

DECLARATION OF DR. VLADIMIR SLEPNEV UNDER 37 C.F.R. 1.132

I declare:

1. I, Vladimir I. Slepnev, hold the position of Chief Scientific Officer at Primera BioSystems, Inc., Mansfield, MA.

2. I hold a Master's Degree in Chemistry from Moscow State University and a Ph.D. in Biochemistry from the Russian Research Center of Molecular Diagnostics and Therapy (Moscow, Russia). I have performed post-doctoral research at Institute Pasteur (France) and at Yale University (New Haven, CT), where I also worked as a junior faculty member.

3. I am an inventor on the above-noted U.S. patent application.

4. I have read the Office Action issued December 27, 2007 in the above-noted patent application, and I understand that the Examiner has rejected claims 1, 4-35 and 38-67 as failing to comply with the enablement requirement. Specifically, the Examiner stated:

> The arguments and declaration argue that capillary electrophoresis, as taught in the prior art by Schumm et al., for example, cannot be used in or adapted to the method of Wiesner, substituting for the slab gel analysis of Wiesner. However, this appears to be exactly what applicant is currently claiming. In other words, the arguments and declaration appear to be arguing that the methods as currently claimed would not work, and thus are not enabled, since it is argued that capillary electrophoresis cannot be used to obtain quantitative information from aliquots of

amplification reactions, and the instant claims are drawn to methods requiring this.

5. I disagree with the Examiner's characterization of the statements made in my declaration filed 7/30/07. I was discussing the state of the art and what Wiesner was teaching. I explained that the methods described by Wiesner are based on and require the use of a linear regression approach to quantitation that will not work if capillary electrophoresis is substituted for the slab gel method taught by Wiesner. Specifically, I explained that the methods described by Wiesner rely "on two key conditions: knowledge of the exact volume of the aliquot subjected to separation, and the ability to measure precisely the absolute quantity of the PCR product band at the given cycle." I explained that "The absolute determination of target template taught by Wiesner will not work when applied to capillary electrophoresis ... because these key conditions will not be satisfied *as required for the linear regression calculations that are* ***central*** *to the Wiesner method*" (emphases added). I also explained that "it is clear from {Wiesner's} description that *the method is based on the exact knowledge of the volume of the aliquot subjected to separation, which becomes the basis for all calculations*" (emphasis added). Thus, I explained that, in order for Wiesner's method to work for quantitation of initial template amounts, one needed to know the volume of the separated aliquot and the amount of nucleic acid in the separated bands, and I explained that when one uses capillary electrophoresis for separation, these two parameters are not available. Thus, one of skill in the art cannot and would not combine separation by capillary electrophoresis with the linear regression approach taken by, and central to, the Wiesner reference to generate quantitative information regarding the abundance of initial templates in a PCR reaction. That is, one of skill in the art cannot combine Wiesner's teachings with separation by capillary electrophoresis and still use the linear regression approach that is central to Wiesner's methods. If one would not be able to use Wiesner's linear regression method to determine initial template amounts (this is the central premise of Wiesner's method), one of skill in the art would not be motivated to use capillary electrophoresis in place of slab gel electrophoresis in Wiesner's method. That is, based on Wiesner's teachings, one would not expect capillary electrophoresis to be applicable for quantitation of initial template amounts, because one would not know the volume of an aliquot separated or the absolute amount of nucleic acid in a separated band.

6. In stark contrast to what Wiesner teaches, we have devised a way to provide quantitative information regarding the initial abundance of template nucleic acids ***without*** *a requirement for* knowledge of the exact volume of the aliquot separated, and ***without*** *reliance* upon the ability to measure precisely the absolute quantity of a PCR product band at a given cycle. Given the teachings of Wiesner, the ability to do so using capillary electrophoresis is surprising and not obvious.

7. I provide data herein demonstrating the use of capillary electrophoresis performed on aliquots taken during a PCR reaction to obtain quantitative information regarding initial template amounts. The approach permits one to obtain quantitative information regarding initial template amounts *without* the need to know either the volume of the aliquot separated or the absolute quantity of a PCR product band at a given cycle. These data were obtained using methods as described in the specification, including the calculation of threshold cycles for the amplified species, and demonstrate that the invention works to provide quantitative information as presently claimed.

6. PCR amplification in exponential phase is commonly described by equation 1:

$$Q_{(C)}= Q_0 * E^C \qquad \text{(equation 1)}$$

wherein $Q_{(C)}$ is the amount of DNA copies at cycle C, Q_0 is the initial number of DNA copies in the reaction and E is efficiency of PCR amplification (number of DNA molecules generated per PCR cycle). Kinetic PCR (such as for example, real-time PCR) uses transformed equation 1 to calculate number of cycles required to a generate fixed (threshold) number of DNA copies.

$T= Q_{(C)}=\text{const}$

$\text{LogT}= \log Q_0 + C_t \log E$ $\qquad C_t$=cycle at threshold T

$Ct= \text{const} - (1/\log E)*\log Q_0$

If one can use experimental methods to assess quantity of DNA wherein the measured signal F is directly proportional to the number of DNA copies in the reaction $Q_{(C)}$,

$$F=f* Q_{(C)},$$

then threshold value can be set in the measured experimental units. Real-time PCR employs measurement of fluorescent signal (generated by fluorescent probes interacting with amplified DNA) calculate the number of cycles required to reach the arbitrary selected threshold of fluorescent signal as a function of initial number of DNA copies in the reaction Q_0. By varying the Q_0 and measuring the Ct corresponding to the given Q_0, one can create a calibration plot and determine copy number in the unknown sample by measuring Ct and correlating it to the Q_0 in the calibration plot.

The use of capillary electrophoresis as an aforementioned physical method to assess quantity of DNA requires: a) that the quantitative fluorescent signal (such as peak height or peak area) is proportional to the amount of DNA molecules, and b) that the amount of injected (and therefore detectable DNA molecules) is proportional to the amount of DNA in the PCR reaction. The first condition requires a consistent way to detect peaks on elecrophoregrams, applying the same rules for background subtraction. The second condition, which is particularly important for electrokinetic injection during CE, assumes that changing representation of different DNA species (such as PCR primers and PCR products) during PCR does not *considerably* affect their injection properties. The validity of these assumptions was demonstrated experimentally (see attached examples).

The methods described and claimed encompass performing PCR amplification, taking a series of aliquots of the PCR reaction (e.g., at every cycle or every other cycle), separating amplified DNA by CE, determining the Ct (number of cycles to reach the threshold fluorescence value selected as log of peak height or peak area expressed in relative fluorescent units) that can be measured for multiple DNA targets simultaneously. By applying different known amounts of DNA targets Q_0 and measuring the Ct corresponding to the given Q_0, one can create a calibration plot and determine copy number in the unknown sample by measuring Ct and correlating it to the Q_0 in the calibration plot.

Described below is an example in which the described method is applied for the simultaneous quantification of several viruses:

A) Template and PCR reaction set up

Template DNA for calibration plot was prepared by spiking plasma matrix with known copies of Cytomegalovirus (CMV), BK virus (BK), and Human Herpesvirus 6 (HHV6B) and Human Herpesvirus 7 (HHV7) at the same copy number for each virus (e.g. 62500 copies for all viruses). The plasma was also spiked with a variable dose of viral particles from 3e6 copies/ml to 3e2 copies/ml for four viruses: Cytomegalovirus (CMV), BK virus (BK), Human Herpesvirus 6 (HHV6b) and Human Herpesvirus 7 (HHV7) so that all four viruses would be present at different copy number (e.g. 62500 copies of BK, 7812.5 for CMV, 976 for HHV6b, 244 for HHV7 in different combinations) to demonstrate linearity of the system. The nucleic acid was extracted from 400ul of the spiked plasma samples with the use of the NucliSENS® easyMAG sample processing instrument and eluted in 25ul of buffer. PCR reactions were set up to quantify the viral targets in the system. The PCR reaction contained; 1.) 2X Qiagen Multiplex PCR master mix, containing HotStarTaq DNA polymerase, Multiplex PCR Buffer, dNTP, $MgCl_2$. 2) 25X Multiplex primer mix, containing a) viral specific primers for each virus tested, primers for the amplification control, 3.) 20ul of extracted sample.

B) Reaction cycling, sampling and separation

The PCR reaction was placed in an MJ research PTC-200 thermocycler that is integrated with a PerkinElmer Multiprobe II Plus liquid handling system. The reaction was amplified under the following conditions: 1) 95°-15minutes, 2) 95° - 30 seconds, 62° - 90 seconds, 72° - 60 seconds – for 3 cycles 3) 95° - 30 seconds, 60° - 90 seconds, 72° - 60 seconds – for 3 cycles 4) 95° - 30 seconds, 58° - 90 seconds, 72° - 60 seconds – for 3 cycles 5) 95° - 30 seconds, 57° - 90 seconds, 72° - 60 seconds – for 33 cycles. Starting at cycle 20, the thermocycler pauses and the lid opened to take a 2ul aliquot of the PCR reaction and dispensed it into 13ul formamide / rox size standards mixture (Bioventures). The integrated thermocycler and liquid handler took an aliquot of the PCR reaction from cycle 18 up to and including cycle 40. After all of the aliquots are taken, the formamide rox mixture that now contained the PCR sample was placed at 95°C for 5 minutes to denature the sample. The denatured samples were loaded into the Applied BioSystems 3730xl DNA Analyzer for analysis of each sample at each cycle taken.

The samples were injected at 2kV for 13 s and separated at 15 kV using a 36 cm 96-capillary array filled with POP7 separating polymer (Applied Biosystems). The fluorescent intensity data for peaks corresponding to target amplicons were extracted from GeneMapper Software

(Applied Biosystems), plotted versus cycle number and subjected to linear regression analysis to identify exponential phase and to calculate Threshold Cycles for target nucleic acids.

C. Data analysis and template quantitation

Data obtained in the exponential phase of the amplification are meaningful for the determination of initial template concentrations. The identification of the exponential phase and Ct calculation can be performed in several different ways (see, e.g., U.S. Patent 6,783,934, "Methods for quantitative analysis of nucleic acid amplification reaction," U.S. Patent 7,228,237, "Automatic threshold setting and baseline determination for real-time," and Zhao S, Fernald R.D., "Comprehensive algorithm for quantitative real-time polymerase chain reaction," J. Comput. Biol. 2005 Oct;12(8):1047-64). The approach we currently use is as follows:

Exponential Phase Identification and Threshold cycle calculation:

Step 1. Enumerate Candidate Point Sets

From the series of fluorescent measurements (RFU units) of increasing cycle number for each target, compile a candidate list of all sets of consecutive measurement points with cardinality 3 or greater.

Step 2. Remove Outliers

Remove internal points from each candidate set whenever the area measurement of a point is less than the area measurement at the previous cycle (typically resulted from error in liquid handling or failed capillary separation) . Candidate sets of cardinality less than 3 are dropped from further consideration.

Step 3. Compute Linear Regression

Compute the best-fitting line for each set using linear regression. Use Log(RFU) and cycle number (C) for each point to create a log-linear amplification curve having equation:

$\text{Log(RFU)} = C0 + E * C$ where C0 is the intercept and E is the slope of the line (Equation 2)

Step 4. Remove Low Quality Sets

Disqualify candidate sets in which the R-squared correlation coefficient value is less than 0.80, the slope (E) is less than 1.45, or the slope is greater than 2.1. If no set passes this quality threshold, then the target is considered not present and reported as not amplified.

Step 5. Compute Fitness Score

The fitness of a candidate set is computed as a weighted sum of quality attributes. These attributes are (1) linearity (R-squared correlation coefficient), (2) number of data points used (cardinality), and (3) proximity to the fluorescence threshold. A set that is closer to the fluorescence threshold is considered higher quality because errors in the linear regression line cause an increase in Ct estimation error that is proportional to distance.

Given an Log(RFU) threshold TRFU, minimum log(RFU) value in the set MinRFU, maximum Log(RFU) in the set MaxRFU, set cardinality Num, and correlation coefficient RSQ, compute the fitness score using Equation 3:

Fitness Score = (Equation 3)

$$RSQ * 100 + \max(5, Num) * 1.0 + \text{if } MinRFU \leq TRFU \leq MaxRFU \text{ then } 1.0$$

else if MaxRFU < TRFU then (TRFU – MaxRFU) * -0.75

else if MinRFU > TRFU then (MinRFU - TRFU) * -0.75

Step 6. Select Best Set

Select the set with the highest fitness score as the best set for the target.

Step 7. Compute Cycle Threshold

From the best set for each target, compute Cycle Threshold (Ct) as the fractional cycle number at the intercept of TRFU on the regression line (defined by Equation 2). Based on comparison of different selected values for TRFU TRFU (Cycle threshold has been chosen at 4.4 units of log peak area corresponding to the middle range of the dynamic range of CE).

Calibration plots Ct versus Cycle number (see appendix A, figure 1) for each virus were constructed based on calculated Ct values for known concentration of the viruses based on the virus particle concentrations provided by the manufacturer (Advanced Biotechnologies). Linear correlation of Ct versus log Q_0 was obtained with R^2 in the range of 0.92-0.98 for different viruses. The generated calibration plots were used for quantification of the set of samples in which viruses were spiked at the different input copy number (the measured Ct for the sample was correlated to the log Q_0 using a calibration plot for corresponding virus). The expected copy number was plotted versus measured copy number demonstrating excellent correlation between two values and validating the use of the described approach to the multiplex quantification of target viruses (appendix A, figure 2). The measured Ct values used for the calibration plot and for linearity experiment are provided in the corresponding tables (Appendix A , tables 1 and 2).

It is emphasized that initial abundance of template nucleic acids was determined *without* a requirement for knowledge of the *exact volume* of the aliquot separated, and *without reliance* upon the ability to measure precisely the *absolute quantity* of a PCR product band at a given cycle.

7. The approach taken to obtain the data described therefore provides quantitative information regarding the abundance of a plurality of different amplification templates in a reaction mixture at the start of the amplification regimen. This approach does not require knowledge of the volume of an aliquot separated or the absolute amount of nucleic acid in a given separated band or peak. The same approach is directly applicable to the quantitative evaluation of RNA in a sample, by adding a step of reverse transcription as described in the specification. An example of using multiplex RT-PCR for quantification of SARS Coronavirus has been published by our group (see a copy of manuscript published in the Journal of Molecular Diagnostics presented as appendix B, figure 6). The method used followed the same steps outlined in the paragraph 6, with few modifications: the reverse transcription step preceded the PCR amplification, the CE analysis was performed on ABI 3100 instrument system using Gene Scan data analysis software (Applied Biosystems), and the Cts were calculated as extrapolation of experimental data from plots of Ct versus input RNA copy number to the log peak area=3. The complete details of the experimental procedures are described in the attached manuscript, which further demonstrates a method in which initial template amounts are determined without

knowledge of the exact volume of the aliquot separated, and without reliance upon the ability to measure precisely the absolute quantity of a PCR product band at a given cycle.

8. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

06/25/08

Date

Vladimir I. Slepnev